*THE BIOTECH GIRL*

# CENTRIFUGATION

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## INTRODUCTION

Centrifugation is the technique which involves the application of centrifugal force to separate particles from a solution according to their size, shape, density, viscosity of the medium and rotor speed.

## BASIC PRINCIPLES

- The principle of the centrifugation technique is to separate the particles suspended in liquid media under the influence of a **centrifugal** field.
- These are placed either in tubes or bottles in a rotor in the centrifuge.
- Particles differing in sizes, shape and density are separated as their sedimentation rate is different.
- The centrifugal force is generated by rotating the rotor of the centrifuge at a high speed. Besides normal and high speed centrifuge there is a very high speed centrifuge known as Ultracentrifuge, which is developed by Theodor Svedberg in 1940. This instrument is designed to produce centrifugal forces up to several hundred thousand times which can separate and purify subcellular organelles, proteins, nucleic acids and several macromolecules. Thus the ultracentrifuge has opened up a new line in many types of fundamental studies in Cell Biology, Biochemistry and Molecular Biology.
- The rate of sedimentation of a particle in a centrifugal force can be shown in the following:

$$\frac{dx}{dt} = \frac{2r^2(P_p - P_m)}{gn} \qquad (1)$$

where $\frac{dx}{dt}$ = the rate at which the particle moves towards the centrifugal field
$r$ = radius of the particle
$P_p$ = densities of the particle
$P_m$ = densities of the medium
$n$ = viscosity of the suspending medium
$g$ = centrifugal force

- Now, the particles will not move if the densities of the particles and the medium are equal.
- **If the densities of the particles are greater than the medium,** they will move toward the bottom of the centrifuge tube while they will remain at the top of the tube, if the particles are lighter than the medium.
- The centrifugal force produced by the centrifuge is measured by the gravity units as:

$$G = w^2 r \qquad (2)$$

where $G$ = centrifugal field
$w$ = angular velocity of the rotor
$r$ = radial distance of the particle from the axis of the rotation

Again, angular velocity of the rotor is known as in the formula:

$$w = \frac{2\text{rev/min}}{60} \qquad (3)$$

Putting the value of w in the equation (2), the centrifugal field (G) will be

$$G = \frac{4^2\ (\text{revolution/min})^2\ r}{60 \times 60}$$

$$\therefore\ G = \frac{4^2\ (\text{rev/min})^2\ r}{3,600}$$

- Which is generally shown as a **multiple of g,** i.e., gravitational field of the earth.
- Sometimes it is also expressed as **R.C.F (Relative Centrifugal Field) which is the ratio of the weight of the particle in the centrifugal field to the weight of the same particle acted on by gravity.**
- On the basis of this principle of separation, particles are separated depending on their densities, size, centrifugal force, time of separation etc.

- **Different cell components are separated in the following order**—whole cells and cell debris first followed by nuclei, plastids, mitochondria, lysosomes, microsomes, fragments of endoplasmic reticulum and ribosome.
- The method of separation becomes complicated when the particles are not spherical, which requires some complicated formula for calculation.
- In case of Ordinary rotors as used in the preparative centrifuge, the centrifugal field does not remain uniform, because the radial dimension of a particle will vary according to the position in the centrifuge tube (rmin and rmax).
- The particle will have a greater centrifugal field as it is further away from the axis of rotation.
- This occurs both in the fixed angle and swing- away rotor. Hence, the centrifugal field is calculated from the average radius of rotation (rav.) of the column of liquid in the centrifuge tube.
- The details of maximum and the method of calculation of relative centrifugal field (R.C.F.) are generally given in the manual of the centrifuge.

The sedimentation rate of a particle can also be expressed as sedimentation coefficient (s) which is the sedimentation rate per unit of centrifugal field. The sedimentation values depend on the solvent-solute systems. The sedimentation coefficient of many of macromolecules is very small. The term s is most often defined under standard conditions, 20°C and water as the medium

- The basic unit is designated as Svedberg unit (S).
- 1 S = $10^{-13}$ Seconds

Sedimentation coefficients of some of the macromolecules are shown in Table

| **Mitochondria** | **Range of sedimentation coefficients in S** |
|---|---|
| Soluble proteins | ? To 25 S |
| Nucleic acids | ? To 100 S |
| Ribosome | 20 to 200 S |
| Viruses | 40-1000 S |
| Mit | 2 * $10^3$ to 70* $10^3$ S |
| Nuclei | 4000 * $10^3$ S to 40,000 * $10^3$ S |

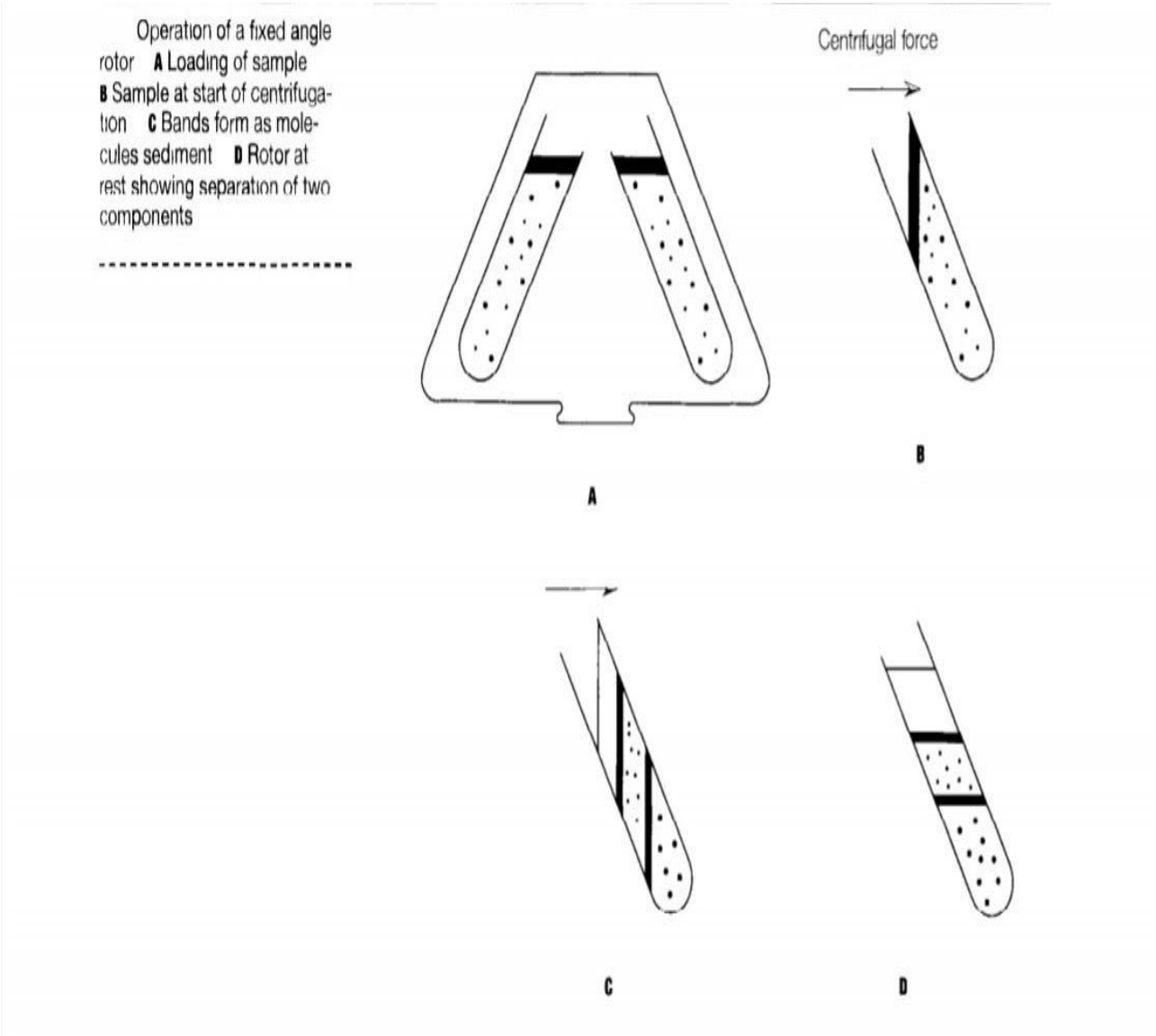
# INSTRUMENTATION FOR CENTRIFUGATION

The basic centrifuge consists of two components :

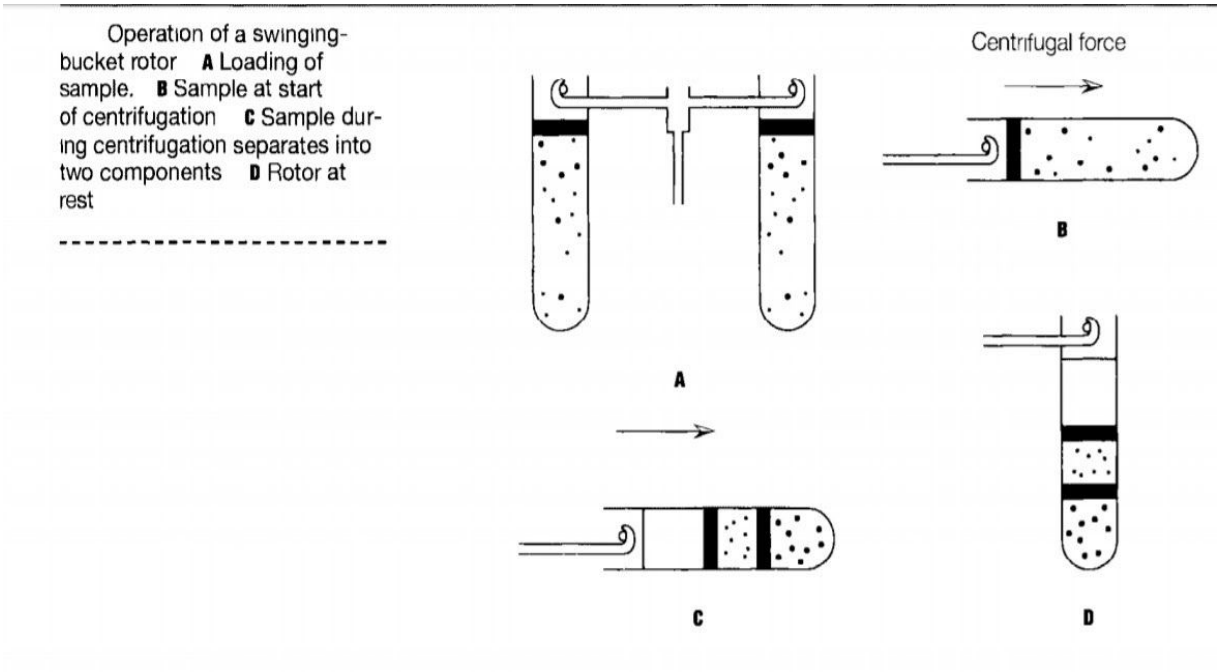
- An **electric motor** with drive shaft to spin the sample
- A **rotor** to hold tubes or other containers of the sample.

Rotor design in centrifuges :

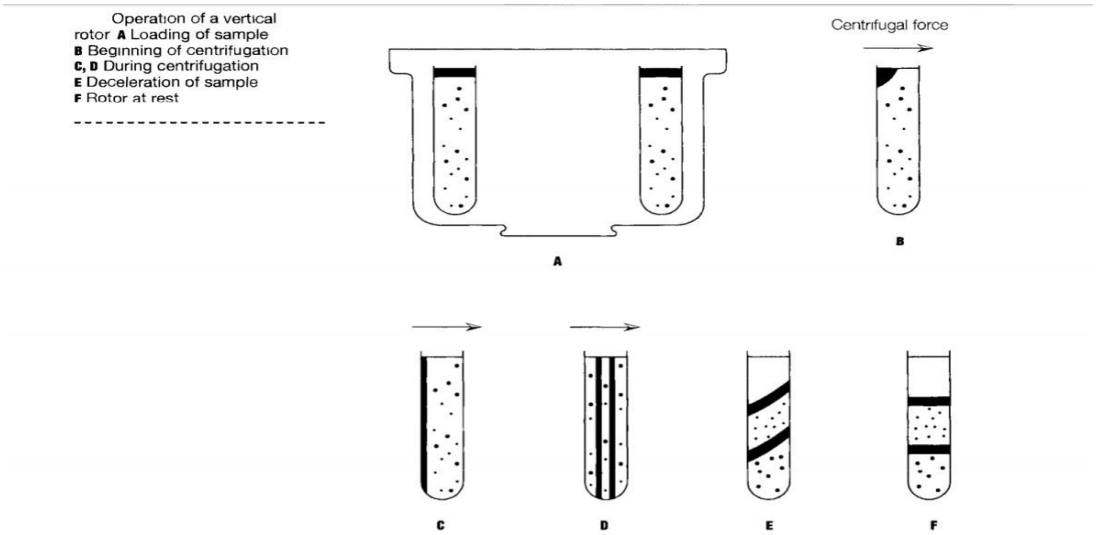
- Fixed angle rotor
    - Useful for differential pelleting of particles.



- Swinging bucket rotor
    - In swinging bucket rotors the samples move to a position perpendicular to the axis of rotation during centrifugation.




- Verticle rotor
    - The sample tubes remain in an upright position.
    - These rotors are used often for density gradient centrifugation.



# TYPES OF CENTRIFUGES

## LOW SPEED CENTRIFUGES

- Used for routine sedimentation of relatively heavy particles .
- The common centrifuge has a maximum speed in the range of 4000-5000 rpm with RCF values upto 3000 g.
- Usually operate at room temperatures.
- No means of Temprature control of the sample.
- Two types of rotors can be used :
    - Fixed angle
    - Swinging bucket
- Centrifuge tubes or bottles that contain **12** or **50** mL of sample are commonly used.
- Low speed centrifuges are especially useful for the rapid sedimentation of coarse precipitates or RED BLOOD CELLS.
- The sample is centrifuged until the particles are tightly packed into a **pellet** at the bottom of the tube.
- The upper liquid portion **the supernatant** is then separated by DECANTATION.

## HIGH SPEED CENTRIFUGES

- The operator of this instrument can carefully **control speed and temprature** which is especially important for carrying out reproducible centrifugation of temprature - sensitive biological samples.
- Rotors chambers in most instruments are maintained at or near **4°C.**
- Three types of rotors are available for high speed centrifugation
    - The fixed angle
    - The swinging bucket
    - The verticle rotor
- HIGH SPEED REFRIGERATED CENTRIFUGES
    - These instruments are used to isolate organelles, to purify and isolate soluble proteins, microorganisms etc. with a speed of about 25,000 r.p.m. having ‘g’ value of 60,000.

    - Both fixed angle and swing-out rotors can be used here. But this speed of the instrument is not sufficient for centrifuging ribosomes and viruses.
- CONTINUOUS FLOW CENTRIFUGE
    - It is also one type of high speed centrifuge where the rotor is slightly modified or specially designed one.
    - In this type there is a continuous flow of the medium in the centrifuge tube. Here the cells or particles axe sedimented against the wall and the excess medium or liquid comes out through the exit tube. Cells can be harvested continuous from a large volume of the culture medium.

## MEDIUM SPEED CENTRIFUGES

- Widely used in this category are **microfuge.**
- Maximum speed - 12,000 - 15,000 rpm
- Delivers force of 11,000 - 12,000 g
- Some instruments can accelerate to full speed in 6 seconds.

The low, medium and high speed centrifuges are of value **ONLY FOR PREPARATIVE WORK THAT IS ONLY FOR ISOLATION AND SEPARATION OF PRECIPITATES AND BIOLOGICAL SAMPLES.**

## ULTRACENTRIFUGES

- HIGH SPEED IS ATTAINED
- Intense heat is generated in the rotor, so spin chamber must be refrigerated and placed under high vacuum to reduce friction.
- Metal rotors when placed in high stress sometimes break into fragments. The rotor chamber on all ultracentrifuges is covered with protective steel armor plate.
- The drive shaft of Ultracentrifuges is constructed of a flexible material to accomodate any wobble of rotor due to imbalance of the samples. It is still important to counterbalance samples as carefully as possible.
- ultracentrifuges can be **USED FOR BOTH PREPARATIVE AND ANALYTICAL MEASUREMENTS.**
- THUS **TWO TYPES** OF ULTRACENTRIFUGES ARE AVAILABLE
    - **PREPARATIVE MODELS** : For separation and purification of samples for further analysis.

- This is a type of instrument where actual isolation, purification of macromolecules or cell organelles can be done. It operates in refrigerated condition under vacuum to avoid frictional resistance of the rotor caused by the spinning air.
- The whole system is sophisticated with continuously monitoring system of the rotor temperature (temperature sensor). There is also one over speed disk system which checks the rotor so that it does not exceed its maximum allowable speed.
- It operates through some photoelectric devices. For ultra smooth and quiet performance of the rotor, it is attached directly to the motor. Most rotors are fabricated from Titanium or Aluminum alloys. Titanium rotor has one advantage that it is quite resistant to corrosion. This instrument can attain a maximum speed of 80,000 r.p.m. and can produce a centrifugal field of 600,000 g.

- **ANALYTICAL MODELS :** for performing physical measurements on the sample during sedimentation.
  - This instrument has many applications in the fundamental studies of macromolecules showing the molecular weight, purity and shape of the material. It runs at a speed of about 70-80,000 r.p.m. with about 500,000 g and consists of a specially designed rotor in a special rotor chamber which remains under vacuum at low temperature.
  - There is an arrangement of a special optical system to determine the concentration distributions within the sample during centrifugation.
  - There are two special optical cells on the rotor, known as the **Analytical cell and the Counterpoise cell.**
  - There are two holes (Reference holes) in the counterpoise cell for the calibration of distances in the analytical cell.
  - The rotor chamber has an upper and lower lens and the upper lens is joined with a camera lens which emits lights on the photographic plate.
  - Light from the light source comes through the bottom.
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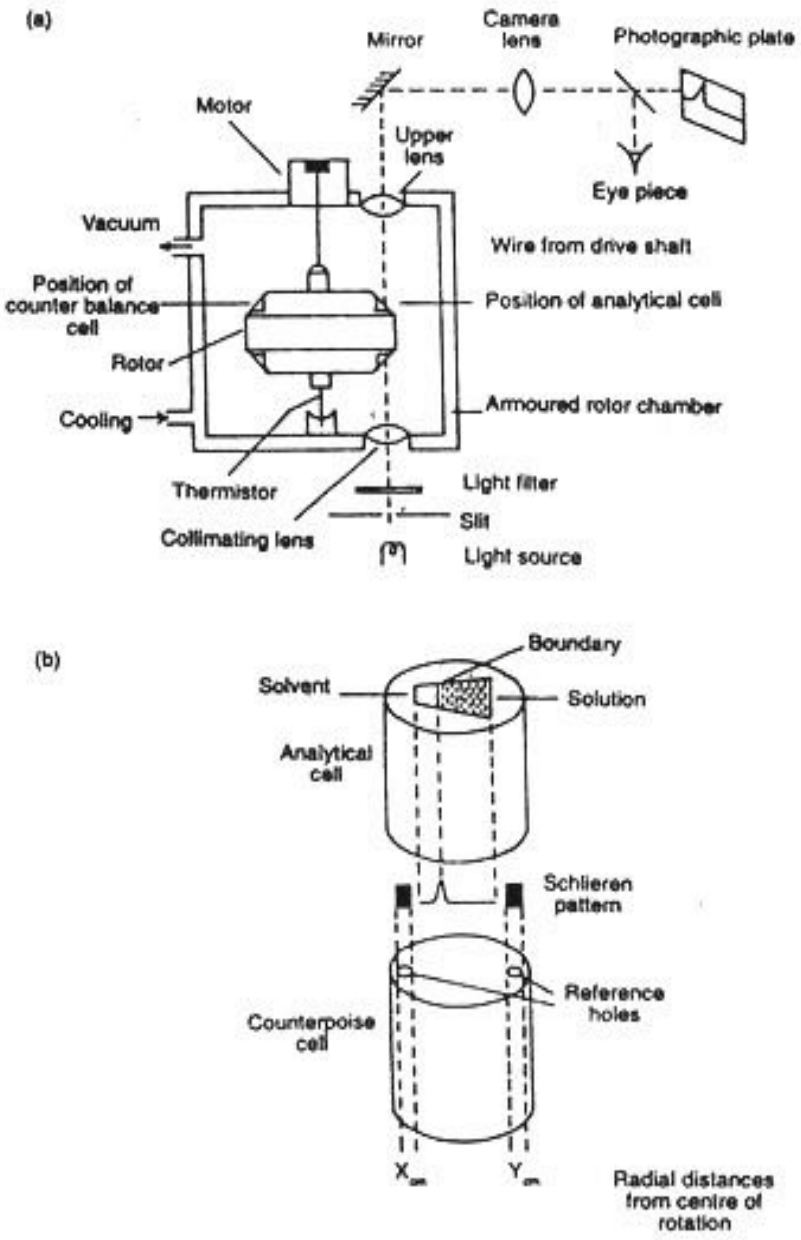


**Fig. 7.2:** Schematic representation of Analytical ultracentrifuge (a) and (b). Details of Analytical and counterpoise cell.

- The principle of monitoring in this system is done either through the ultraviolet absorption system or by noting the differences in the refractive index. If the concentration is uniform, light passes through it without any

deviation. But if the light passes through a solution of different density zones, it is refracted at the boundary between these zones.

- By measuring the refractive index between the reference solvent and the solution, the concentration of solute at any point can be measured.
- In recent models, the photographic plate system has been replaced by electronic scanning system which can directly measure and plot the concentration of the sample at all points in the analytical cell.
- Centrifuge tubes are either manufactured in hard glass or with polypropylene, polycarbonate, stainless steel and nylon materials.
- Generally, centrifugation is done to separate the particles or cells on the basis of their size, length or mass. But in some cases, separation is done on the basis of the density of the particles.
- When the shape (size) and density of some macromolecules are same then these macromolecules can be separated from each other according to mass. This type of separation through centrifugation is known as Rate Zonal Centrifugation. As the name signifies, different-sized molecules will occupy different zones in a centrifuge tube after centrifugation.

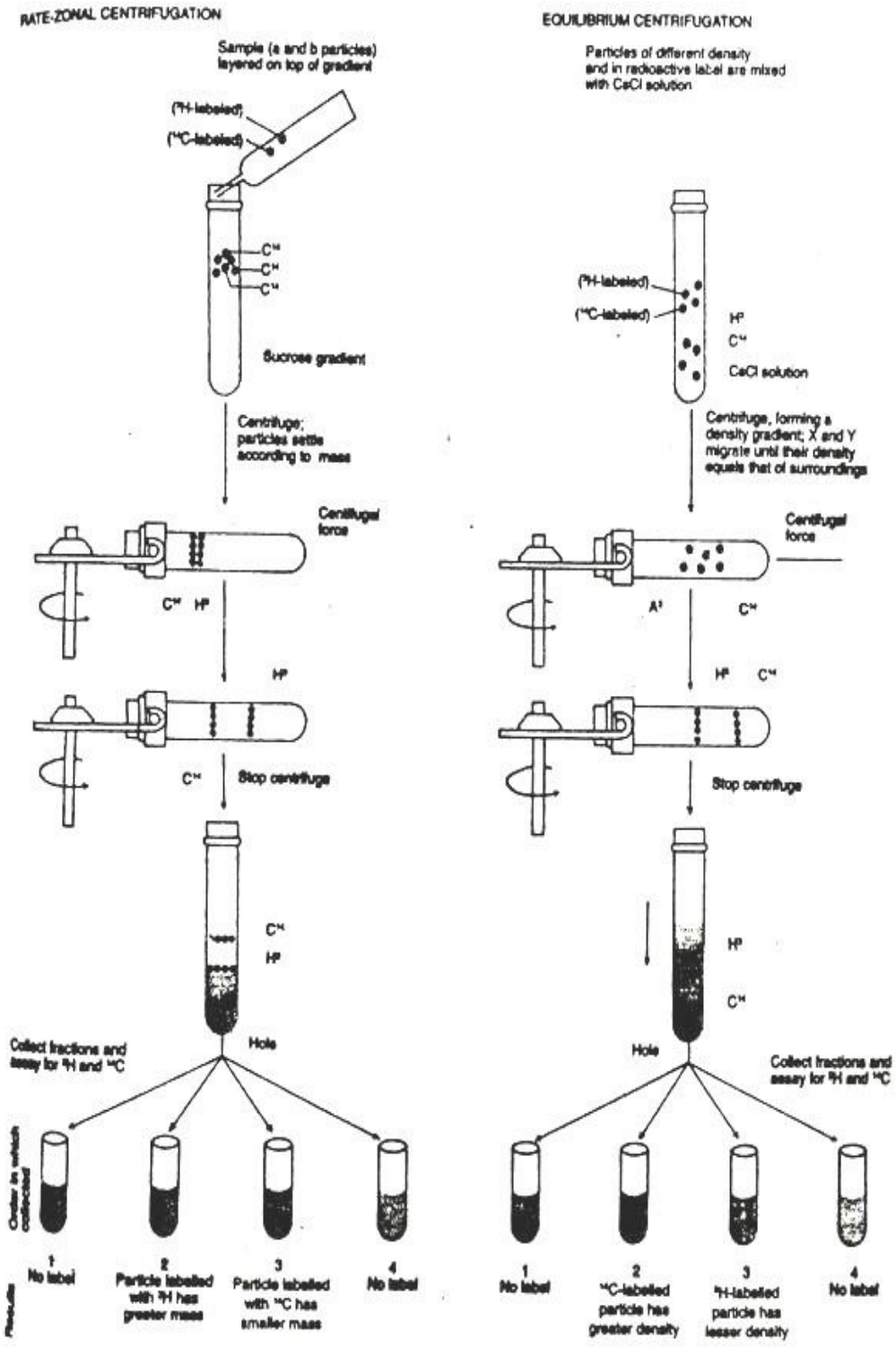


Fig. 7.4: Separation process: (a) by Rate-Zonal centrifugation, and (b) by Equilibrium centrifugation.

- This separation of macromolecules at different zones is stabilized by using Sucrose gradient. So, this technique is also known as **Sucrose Density Gradient Centrifugation.** This is used for separating all types of particles and organelles.
- In analytical ultracentrifuge the rate of sedimentation can be measured by taking photographs of the moving boundaries of sedimenting particles.
- To separate particles of different densities, they are labelled with H3 or C14 and then centrifuged in Sucrose density gradient. Particles labelled with H3 have the greater mass and sediment faster than those labelled with C14.
- The second type of density gradient centrifugation is known as **Equilibrium Density Gradient Centrifugation** where the density gradient is formed during centrifugation. The material used in this process is the aqueous solution of Caesium chloride (CsCl).After a run in the ultracentrifuge, it will form 0.92g/ml heavier at the bottom than at the top permitting the separation of particles which differ in density by even a fraction of 0.02g/ml.The densities of protein, DNA and RNA are 1.3, 1.6 to 1.7 and 1.75 to 1.8 g/ml, respectively. The same chemical CsCl can separate the different macromolecules like DNA and RNA due to the fact that Cs+ binds to DNA at phosphate groups, while it binds to RNA both at phosphates and at the hydroxyl groups of sugar thus increasing the density of RNA more than that of DNA.
- Different isotopes are used with different labelling precursors to alter their densities which will be helpful for the separation of these macromolecules on the basis of density.
- Gradient forming chemicals commonly used are Caesium and Rubidium chloride, Sucrose, some proteins and polysaccharides, colloidal silica (Percoll, Ludox), Metrizamide, Nycodenz, Renograffin etc.

| **CHEMICALS** | **IONIC STRENGTH** | DENSITY OF AQUEOUS SOLUTION | **COMMON USES** |
|---|---|---|---|
| CAESIUM CHLORIDE | HIGH | 1.91 | Separating DNA, Nucleoproteins, viruses, isolation of plasmids |
| CAESIUM BROMIDE | HIGH | 2.01 | Separation of DNA, RNA etc |
| SODIUM BROMIDE | HIGH | 1.53 | Fractionation of Lipoproteins |
| GLYCEROL | NON IONIC | 1.26 | Separation of membrane fragments |
| SUCROSE | NON IONIC | 1.32 | Separation of subcellular particles |
| FICOLL | NON IONIC | 1.17 | Separation of cells, nucleic acids, organelles |
| DEXTRAN | NON IONIC | 1.13 | Cells, microsomes |
| PERCOLL | NON IONIC | 1.30 | Cells and organelles |
| METRIZAMIDE | NON IONIC | 1.46 | Cells, organelles, nuclei membranes |
| NYCODENZ | NON IONIC | 1.42 | Cells, organelles, Nucleoproteins, viruses |

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    - Of them, Sucrose is most commonly used in Density Gradient centrifugation because of being very viscous even at 10% concentrations. Ficoll (copolymer of Sucrose and epichlorhydrin) is used for the separation of whole cells and cellular organelles.
    - CsCl solutions are used for Equilibrium Density Gradient or isopycnic separation of nucleic acids. After density gradient centrifugation, generally the visual bands separating the particles are collected with the help of the hypodermic needle or syringe.
    - Sometimes the centrifuge tube is punctured at the base by a fine needle. As the drops of the liquid come out through the needle they may be collected and analysed using ultraviolet spectrophotometer
- One of the most versatile model is **BECKMAN TLX,** A microprocessor controlled tabletop Ultracentrifuge. With a typical fixed angle rotor which holds six,  0.2 to 2.2 mL samples, the instrument can generate 100,000 rpm and generates upto 600000g. The beckman instrument can scan the sample over the wavelength range 190-800 nm.

# METHODS FOR SEPARATION OF PARTICLES IN CENTRIFUGATION

The three methods are:

- Differential Centrifugation
- Centrifugal Elutriation
- Density Gradient Centrifugation
  - Rate Zonal Technique
  - Isopycnic Centrifugation Technique

## DIFFERENTIAL CENTRIFUGATION

- This depends upon the sedimentation rate of particles of different size and density. **Centrifugations will initially sediment the largest particles.**
- For particles with same mass but with different densities, **the one with highest density will sediment first.**
- Particles having similar banding densities can usually be efficiently separated one from another by differential centrifugation or rate zonal method, provided that there are at least 10-fold differences in their masses.
- In differential centrifugation the material to be separated is divided centrifugally into number of fractions by **increasing the applied centrifugal field.** The centrifugal field at each step is chosen so that particular type of material sediments.
- Any type of particle originally present in homogenate may be found in pellet or the supernatant or both fractions, depending upon the time and speed of centrifugation and size and density of particles.
- At the end of each stage the pellet and supernatant are separated and pellet washed several times by re-suspension and re-centrifugation in homogenation medium.
- Initially all particles of homogenate are homogenously distributed throughout the centrifuge tube. During centrifugation particles move down the centrifuge tubes

at their respective sedimentation rates and start to form a pellet on the bottom of centrifuge tube. Ideally centrifugation is continued enough to pellet all the largest class of particles, the resulting supernatant then being centrifuged at a higher speed to separate medium-sized particles and so on.

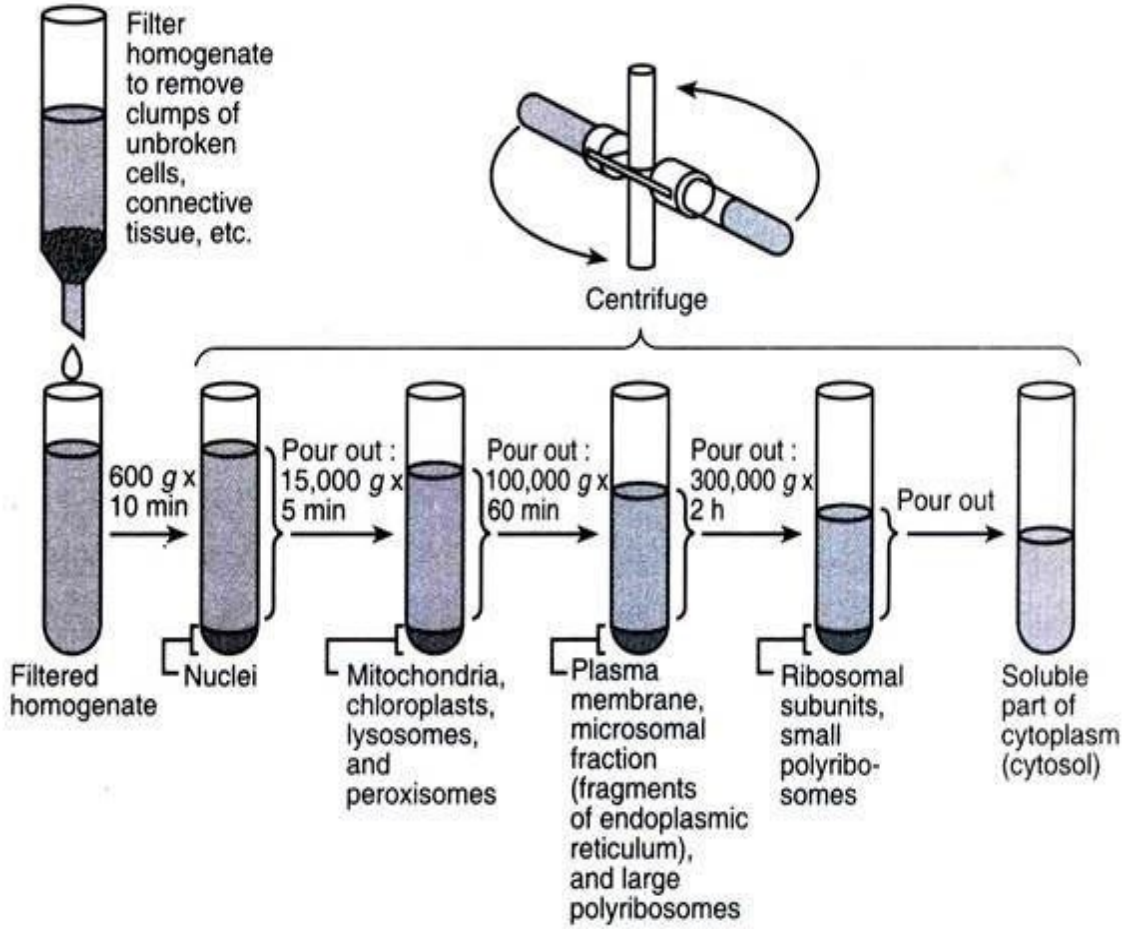


**Fig. 5.13:** Isolation of different cell organelles by differential centrifugation

- However, since particles of varying sizes and densities were distributed homogenously at the commencement of centrifugation, it is evident that the **pellet will not be homogenous but will contain a mixture of all the sedimented components, being enriched with fastest sedimenting particles.** In the time required for complete sedimentation of heavier particles, some of the lighter and medium sized particles, originally suspended near the bottom of the tube, will also sediment and thus contaminate the fraction.

- Pure preparation of the pellet of the heaviest particle cannot be, therefore, obtained in single centrifugation step. It is only the most slowly sedimenting component of mixture remaining in the supernatant after all the larger particles have been sedimented that can be purified by single centrifugation step.
- The separation achieved by differential centrifugation can be **improved by repeated re-suspension and re-centrifugation under similar condition**.
- Further centrifugation of the supernatant with **gradually increasing centrifugal fields** results in sedimentation of intermediate and finally the smallest and least dense particles.
- In spite of its inherent limitations, differential centrifugation is probably the most commonly employed method for isolation of cell organelles from, homogenized tissue.

## CENTRIFUGATION ELUTRIATION

- In this technique the separation and purification of a large variety of cells from different tissues and species can be achieved by a gentle "**washing action"** using an elutriator rotor.
- The technique is based upon the differences in the set-up in separation chamber of rotor, between the opposing centripetal liquid flow and applied centrifugal field being used to separate particles mainly on the basis of differences in their size.
- The technique does not employ the density gradient and have advantage that any medium totally compatible with the particles can be used.
- By this, separation can be achieved very quickly, giving high cell concentrations and a very good recovery yield.

## DENSITY GRADIENT CENTRIFUGATION

- There are two methods of density gradient centrifugation, the rate zonal technique and the isopycnic (iso-density or equal density) technique, and both can be used when **quantitative separation of all the components of mixtur**e of particles is required.

- They are also used for the determination of buoyant densities and for the estimation of sedimentation coefficient
- **RATE ZONAL TECHNIQUE :**
    - Particle separation by the rate zonal technique is based upon **differences in the size, shape and density of particles, the density and viscosity of the medium and the applied centrifugal field.**
    - Subcellular organelles, which have different densities but are similar in size, do not separate efficiently using this method, but separation of proteins of similar densities and differing only 3 folds in relative molecular mass can be achieved easily.
    - The technique involves carefully layering a sample solution on top of preformed liquid density gradient, the highest density of which does not exceed that of densest particle to be separated.
    - The function of gradient is primarily to stabilize the liquid column in the tube against the movements resulting from conventional currents and secondarily to produce a gradient that helps to improve the resolution of gradient.
    - The sample is then centrifuged until the desired degree of separation is achieved. Since the technique is time dependent, centrifugation must be terminated before any of the separated zone pellets at he bottom of tube.
    - The technique is employed for the separation of enzymes, RNA-DNA hybrids, ribosomal subunit, subcellular organelle, etc.

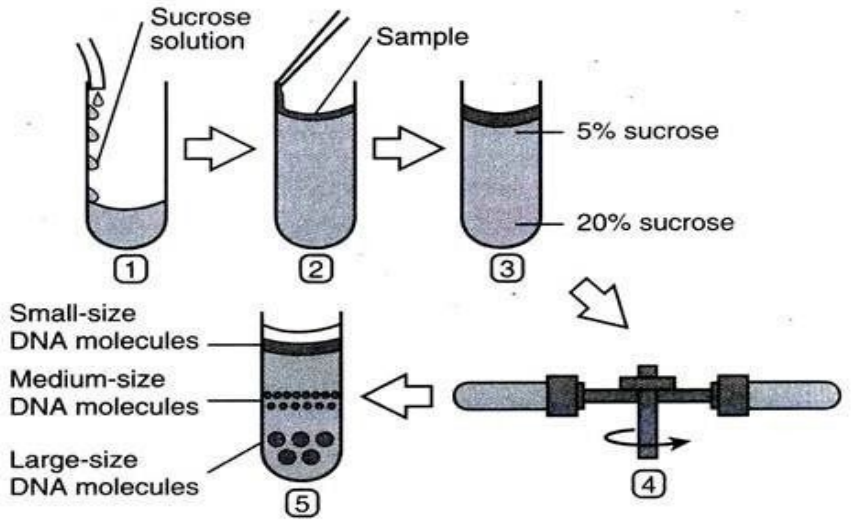


**Fig. 5.14:** Principle of rate zonal centrifugation technique

- ISOPYCNIC CENTRIFUGATION TECHNIQUE
    - Isopycnic centrifugation depends solely upon the **buyout density and not on its shape, size and time,** the size of the particle affecting only the rate at which it reaches its isopycnic position in the gradient.
    - The technique is used to separate particles of similar size but of different density.
    - Hence soluble proteins which have very similar densities cannot be usually separated by this method, where as sub cellular organelles can be effectively separated.

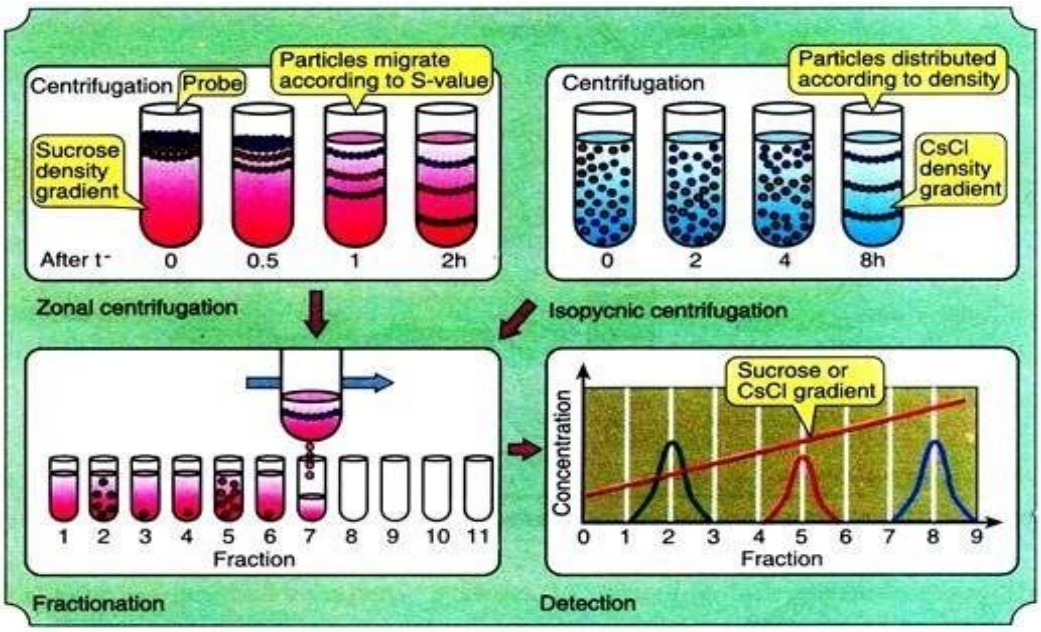


**Fig. 5.15:** Density gradient centrifugation

- The methods are a combination of sedimentation and flotation and involve layering the sample on top of a density gradient that spans the whole range of the particle densities that are to be separated. The maximum density of the gradient, therefore, must always exceed the density of the densest particle. During centrifugation, sedimentation of the particle occurs until the buoyant density of the particle and density of the gradient are equal.
- At this point of isodensity no further sedimentation occurs, irrespective of how long centrifugation continues, because the particles are floating on the cushion of material that has density greater than their own. Isopycnic centrifugation, in contrast to the rate zonal technique, is an equilibrium method, the particle banding to form zones each at their own characteristic buoyant density.

- In case when not all components in a mixture of particle are required, a gradient range can be selected in which unwanted materials will be sediment at the bottom of the tube and whole of the particles of interest will float at their respective isopynic positions. Such a technique involves a combination of both the rate zonal and isopycnic approaches.

| Material | Density ( g/cm ³) |
|---|---|
| Microbial cells | 1.05 TO 1.15 |
| Mammalian cells | 1.04 TO 1.10 |
| Organelles | 1.10 TO 1.60 |
| Proteins | 1.30 |
| DNA | 1.70 |
| RNA | 2.00 |

- FEATURES OF GRADIENT MATERIAL :
  - **There is no ideal all purpose gradient material; the choice of solute depends upon the nature of the particles to be fractionated**
  - The gradient material should
    - Permit the desire type of separation
    - Be stable in solution
    - Be inert towards biological material and not react with the centrifuge , rotor, tubes or caps:
    - Not absorb light at wavelengths appropriate for spectrophotometric monitoring (visible or ultraviolet range), or otherwise interfere with assaying procedures
    - Be sterilisable, non-toxic or flammable;
    - Have negligible osmotic pressure and cause minimum changes in ionic strength, pH and viscosity;
    - Be inexpensive and readily available in pure form and capable of forming a solution covering the density range

needed for a particular application without overstressing the rotor;

- Allow easy separation of the sample material from the gradient medium with loss of the sample or its activity.

- **Generally used gradient materia**ls are salts of alkali metals (e.g., caesium and rubidium chloride), small neutral hydrophilic organic molecules (e.g., sucrose), hydrophilic macromolecules (e.g., proteins and polysaccharides), and a number of miscellaneous compounds such as colloidal silica (e.g., percoll and ludox) and non-ionic iodinated aromatic compounds (e.g., metrizamide, nycodenz and renograffin).

- Sucrose solution while suffering from disadvantages of being very viscous at densities greater than 1.1 to 1.2 g cm-3 and exerting very high osmotic effects even at very low concentrations have been found to be most convenient gradient material for rate zonal separation.

- Glycerol is also used as gradient material especially for the separation of enzymes, or alternative media such as ficoll, metrizamide or percoll gradients may be utilized.

- Non-ionic media, such as sucrose, glycerol, metrizamide, ficoll and percoll are generally considered to be gender than ionic salts like caesium chloride and potassium bromide and require a lower centrifugal fields to achieve adequate separation of particles. In case of isopycnic separation, no one medium has proved satisfactory for the isolation of all type of biological particles.

- Ficoll has successfully used for the separation of whole cell and subcellular organelles by rate zonal and isopycnic centrifugations. Caesium and rubidium salts are used exclusively for isopycnic separations and have been used most frequently for separation of high density solutes like nucleic acids.

- However, at high concentrations their high ionic strength and osmolarity tends to disrupt intra- and inter-molecular bonds. Generally, ionic media have been used for the separation of nucleic acids, proteins and viruses and non-ionic iodinated aromatic compounds, because of their increased versatility, have been used for a much wider variety of applications.

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| Gradient media | Cells | Viruses | Organelles | Nucleoproteins | Macromolecules |
|---|---|---|---|---|---|
| **Sugars** | + | +++ | +++ | + | - |
| **Polysaccharides** | ++ | ++ | ++ | - | - |
| **Colloidal silica** | +++ | + | +++ | - | - |
| **iodinated media** | ++++ | ++ | ++++ | +++ | + |
| Alkali metal salts | - | ++ | - | ++ | ++++ |

Application of density gradient media for isopycnic separations

++++ → Excellent

+++ → Good

++ → Good for some applications

+ --> limited use

- → unsatisfactory

## APPLICATIONS OF CENTRIFUGATION

- Centrifugation can be employed to separate a mixture of two different miscible liquids.
- This technique can also be used in order to study and analyze macromolecules and their hydrodynamic properties.
- Mammalian cells can be purified with the help of a special type of centrifuge.
- Centrifugation is known to have a vital role in the fractionation of many subcellular organelles. Furthermore, centrifugation is also useful in the fractionation of membrane fractions and membranes.
- Centrifugation also has applications in the fractionation of membrane vesicles.
- Skimmed milk is a form of milk that has a lower amount of dissolved fats. Skimmed milk can be obtained from regular milk with the help of the process of centrifugation. Here, the centrifuge serves to separate the fat from the milk, leaving the required skimmed milk behind.
- Cyclonic separation is an important process that has vital applications in the separation of particles from air flows.
- Another important application of this technique is in the stabilization and clarification of wine.
- This technique, in combination with other purification techniques, is extremely helpful while separating proteins. Other techniques that are used include salting out techniques such as ammonium sulfate precipitation.
- Centrifuges are widely used in the field of forensic chemistry. In this field, the technique is employed for the separation of blood components from blood samples. Furthermore, the technique is also employed in certain laboratories for the separation of urine components from urine samples.
- Differential centrifugation, a distinct type of centrifugation, is known to have applications in the identification of organelles.

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## SOURCES


- BIOLOGY DISCUSSION
- WIKIPEDIA
- MODERN EXPERIMENTAL BIOCHEMISTRY